Zasp regulates integrin activation

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Summary

Integrins are heterodimeric adhesion receptors that link the extracellular matrix (ECM) to the cytoskeleton. Binding of the scaffold protein, talin, to the cytoplasmic tail of β-integrin causes a conformational change of the extracellular domains of the integrin heterodimer, thus allowing high-affinity binding of ECM ligands. This essential process is called integrin activation. Here we report that the Z-band alternatively spliced PDZ-motif-containing protein (Zasp) cooperates with talin to activate α5β1 integrins in mammalian tissue culture and αPS2βPS integrins in *Drosophila*. Zasp is a PDZ–LIM-domain-containing protein mutated in human cardiomyopathies previously thought to function primarily in assembly and maintenance of the muscle contractile machinery. Notably, Zasp is the first protein shown to co-activate α5β1 integrins with talin and appears to do so in a manner distinct from known αIIbβ3 integrin co-activators.

Key words: PDZ domain protein, Zasp, Adhesion receptor, Extracellular matrix, Integrin activation, Muscle attachment

Introduction

Tight coordination of cell–extracellular matrix (ECM) adhesion is essential throughout metazoan development and in adult organisms. Integrins are the major family of ECM adhesion receptors, and regulation of integrin affinity for ligand (integrin activation) is a key control point in cell adhesion, migration and assembly of the ECM (Calderwood, 2004). Consistent with its central role in these fundamental processes, perturbation of integrin activation in model organisms impairs viability and disrupts development, tissue formation, morphogenesis, cell trafficking and mechanosensing (Czuchra et al., 2006; Kendall et al., 2011; Martin-Bermudo et al., 1998; Millon-Frémillon et al., 2008; Pines et al., 2011; Tanentzapf and Brown, 2006).

Integrin activation occurs when intracellular signals impinge upon the usually short (~50 amino acids) integrin cytoplasmic tails, causing conformational rearrangements of integrin extracellular domains (Shattil et al., 2010). It is now known that binding of the cytoskeletal adaptor protein, talin, to the cytoplasmic tail of integrin-β subunits is a crucial step in integrin activation, and models for how talin impacts integrin activation have been proposed (Bouaouina et al., 2008; Harburger and Calderwood, 2009; Kim et al., 2012; Moser et al., 2009b; Shattil et al., 2010; Tadokoro et al., 2003; Ye et al., 2010). However, it has recently been appreciated that additional factors control integrin activation *in vivo*, and identification of these factors will be required for a complete understanding of the molecular basis of integrin activation (Harburger and Calderwood, 2009; Moser et al., 2009b; Shattil et al., 2010). Some known integrin activators, such as Rap1, act via talin, as RIAM, the effector of Rap1, causes membrane targeting of talin and thereby enhances integrin activation (Han et al., 2006; Lee et al., 2009). Other integrin activators, such as the kindlins, must directly bind integrin-β tails in order to enhance talin-mediated activation, and we and others have shown that co-expression of kindlin-1 or -2 with the head domain of talin potentiates talin-mediated activation of αIIbβ3 integrins (Harburger et al., 2009; Ma et al., 2008; Montanez et al., 2008; Moser et al., 2009a; Moser et al., 2009b; Moser et al., 2008). However, in Chinese hamster ovary (CHO) cells, co-expression of kindlin-1 or -2 with talin head fails to activate β1 integrins, indicating that alternative co-activating factors may exist for β1 integrins (Harburger et al., 2009). Here we combine *in vivo* studies in *Drosophila* with mammalian cell culture experiments to show that the PDZ–LIM-domain-containing protein, Zasp, cooperates with talin to activate β1 integrins.

Zasp (or Cypher in mouse) belongs to the Alp/Enigma protein family, members of which have one N-terminal PDZ domain and up to four C-terminal LIM domains (Te Velthuis et al., 2007). Mammalian Zasp isoforms are mainly expressed in muscle as a prominent component of sarcomeric Z-lines, functioning in the assembly and maintenance of the muscle contractile machinery. While Zasp lacks enzymatic activity, it can act as an adaptor protein, binding α-actinin-2 to stabilize Z-lines in striated and cardiac muscle (Faulkner et al., 1999; Zhou et al., 2001; Zhou et al., 1999). Mutated Zasp can lead to the development of hypertrophic cardiomyopathies and myofibrillar myopathies (Sheikh et al., 2007). Mammalian Zasp is subject to extensive alternative splicing with up to six protein variants expressed, but all mammalian Zasp isoforms are composed of an N-terminal PDZ domain followed by a Zasp-like motif (ZM), an intervening sequence of variable length and no, one or three C-terminal LIM domains (Fig. 1A) (Faulkner et al., 1999; Vatta et al., 2003).

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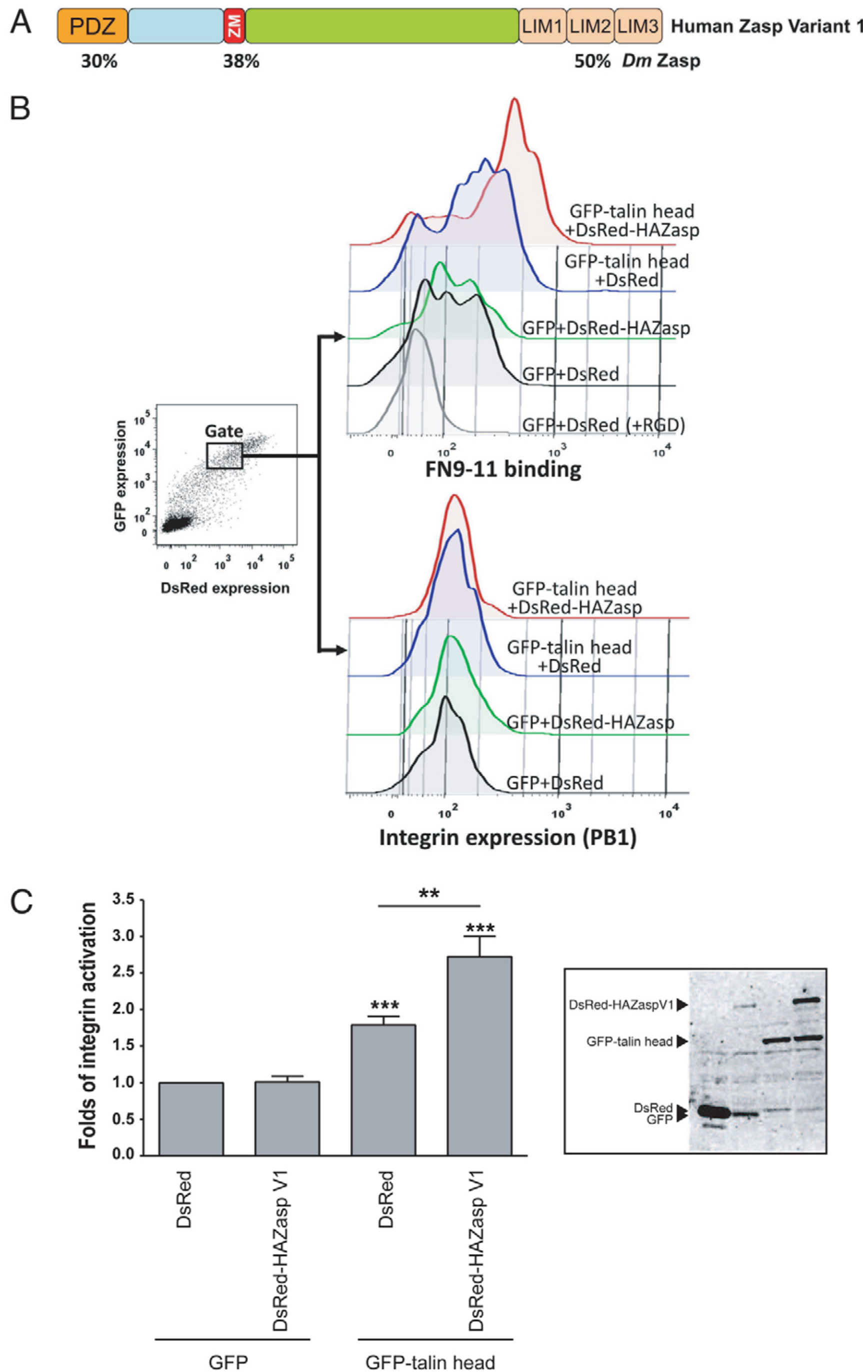

Fig. 1. Human Zasp activates α5β1 integrins. (**A**) Schematic diagram of human Zasp variant1 (Zasp V1, 727 amino acids). The percentage amino acid identity between the three conserved domains of hZasp and *Drosophila* Zasp (*Dm* Zasp) is given below. (**B**) Flow cytometry data analysis of CHO cells transfected with DNA encoding GFP and/or DsRed-tagged talin head and Zasp V1 proteins. A gate was drawn to define a double positive (GFP and DsRed) population. Histogram plots from cells in this gate were generated to measure the geometric mean fluorescence intensity of GFP, DsRed, and FN9-11 or PB1. Activation of endogenous α5β1 integrin in CHO cells co-expressing GFP or GFP–talin head and DsRed or DsRed–HAZasp V1 was calculated as described in Materials and Methods. (**C**) Talin head and hZasp synergistically activate α5β1 integrin in CHO cells. Results represent mean ± s.e.m. ($n \geq 3$; $^{**}P<0.01$ and $^{***}P<0.001$). Inset shows expression of transiently transfected proteins.

Drosophila Zasp is spliced even more extensively, with 13 splice variants documented so far (Katzemich et al., 2011).

We previously reported that Zasp is involved in the assembly of integrin adhesion sites in *Drosophila* muscle, that Zasp genetically interacts with αPS2 integrin during muscle attachment, and that in Zasp-deficient flies, embryonic and first larval instar muscles partially detach from myotendinous junctions (Jani and Schöck, 2007). However, it was unclear how Zasp impacts integrin-mediated adhesion and whether it is required to maintain the integrin–cytoskeletal link for adhesion to the ECM. Here we describe an unanticipated role for Zasp in regulating integrin activation.

Results

Mammalian Zasp cooperates with talin head to activate α5β1 integrins

The Zasp-deficient *Drosophila* phenotype closely resembles and manifests itself concurrently with the previously reported talin-mutant phenotype, in which an R367A point mutation of the talin head domain disrupts integrin activation (Tanentzapf and Brown, 2006). The similarity in phenotype of Zasp-deficient and talin-mutant flies prompted us to directly assess the ability of human Zasp to trigger activation of mammalian integrins. We accomplished this using a dual color flow cytometric assay that measures binding of a purified, recombinant fragment of

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fibronectin (FN9-11) to activated α5β1 integrins in transfected CHO cells (Bouaouina et al., 2012; Harburger et al., 2009). The assay is normalized to surface integrin expression using anti-α5β1 integrin antibodies that bind in an activation-independent manner, and cells are gated to have an equivalent level of transfected fluorescently tagged protein (Fig. 1B). Transient expression of DsRed–HA-tagged human Zasp variant 1 (Zasp V1) in CHO cells does not significantly alter α5β1 integrin activation compared to the GFP and DsRed control; however, co-expressing DsRed–HAZasp V1 and GFP-tagged talin head significantly increases α5β1 integrin activation above the levels induced by GFP–talin head alone (Fig. 1C). Integrin activation in this assay is cell autonomous, as only transfected cells are activated, and neither GFP nor DsRed alone have an effect on integrin activation. Thus, expression of Zasp in CHO cells potentiates talin-head-mediated α5β1 integrin activation, indicating that Zasp can modulate integrin activation.

Zasp deficiency in *Drosophila* muscles causes detachment of integrins from the ECM

Our finding that mammalian Zasp enhances integrin activation in cultured cells prompted us to test whether Zasp also plays a role in integrin activation *in vivo*, using *Drosophila* as a model organism. There is no well-established assay in *Drosophila* to measure integrin activation, but it has been reported that in a talin head mutant, integrins separate from the ECM at the myotendinous junction (Tanentzapf and Brown, 2006). We first confirmed that Zasp colocalizes with talin at myotendinous junctions (Fig. 2). We further found that in Zasp-deficient *Drosophila* embryos, αPS2 integrins still localize to the ends of detached body wall muscles but exhibit a partial separation from the ECM ligand, tiggrin, indicating that Zasp regulates integrin adhesion to *Drosophila* ECM (Fig. 3). This further suggests that Zasp plays a role in integrin activation in *Drosophila*, and that integrin activation is important for the maturation of myotendinous junctions. This effect is not due to an alteration of talin localization, as talin is still found at the ends of detached muscles along with αPS2 integrin in *Zasp* mutant embryos (Fig. 3).

Integrin mobility is higher in myotendinous junctions of *Zasp* mutant larvae

It was recently shown by fluorescence recovery after photobleaching (FRAP) experiments that integrin turnover and mobility correlate with the strength and stability of myotendinous junctions (Yuan et al., 2010). In newly attached muscles, integrin mobility is very high and is considerably reduced during myofibril maturation, presumably to withstand the increased contractile forces acting on myotendinous junctions (Yuan et al., 2010). We therefore asked how integrin mobility develops in *Zasp* mutants. While wild-type embryos and first instar larvae show a consecutive decrease of integrin mobility, integrin mobility stays essentially the same throughout myotendinous junction maturation in *Zasp* mutants (Fig. 4A). A change in integrin mobility between wild-type and *Zasp* mutants is first observed in stage 17 embryos, when we also first observe the *Zasp* mutant phenotype of muscle detachment (Jani and Schöck, 2007). Importantly, talin head mutants (talinR367A) show a similar increase in integrin mobility in stage 17 embryos (Fig. 4B), demonstrating that defects in integrin activation are associated with increases in integrin mobility. These data indicate that integrin affinity to the ECM or to the actin cytoskeleton is lower in *Zasp* mutant stage 17 embryos and first instar larvae, resulting in muscle detachment when muscle contractility begins.

Recently several point mutations in the extracellular domain of βPS integrin were identified that increase the affinity of αPS2βPS integrin binding to the extracellular matrix and cause lethality owing to this increase in affinity (Kendall et al., 2011). Lethality associated with some of these mutations can be suppressed by removing one copy of talin, confirming the role of talin as an integrin activator (Kendall et al., 2011). We therefore tested the genetic interaction of two of these mutants (b44, I375F in the ADMIDAS and b30, I298F in β-I domain) with *Zasp*. Removing one copy of *Zasp* increases viability of b44 from 33% viable mutant males to 54%, $n=1445$ [21% to 59% for $rhea^2$ (*Drosophila* talin) (Kendall et al., 2011)] and it increases viability of b30 from 41% to 57%, $n=1516$ [3% to 44% for $rhea^2$ (Kendall et al., 2011)]. This suggests that both talin and Zasp are involved in modulating integrin affinity in *Drosophila*.

Ectopic expression of talin head partially rescues the *Zasp* phenotype

Further support for a link between Zasp and integrin activation comes from the ability of a talin head transgene to partially suppress the lethality associated with the *Zasp* mutant phenotype (Fig. 4C). Expression of UAS–talin head in muscles with Dmef2–Gal4 results in its localization to myotendinous junctions, but does not cause dominant-negative effects (Tanentzapf et al., 2006). Furthermore, talin head localizes normally to muscle ends in both wild-type and *Zasp* mutant larvae (supplementary material Fig. S1). We therefore expressed this transgene in muscles in homozygous *Zasp* mutants, and assessed their viability compared to *Zasp* mutant controls expressing the UAS–talin head R367A mutant. Notably, homozygous *Zasp* mutant larvae expressing UAS–talin head live significantly longer on average (Fig. 4C), showing that UAS–talin head can partially suppress *Zasp* mutant phenotypes. In contrast, overexpressing Zasp does not cause any observable rescue of *talin* null mutant flies (data not shown). Given that the major described function of talin head is integrin activation, and

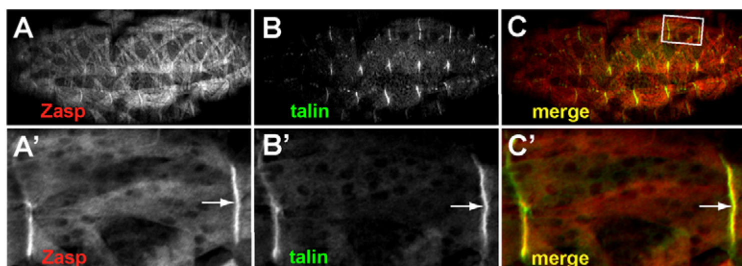

Fig. 2. *Drosophila* Zasp and talin co-localize at myotendinous junctions. (A–C′) Zasp colocalizes with talin at myotendinous junctions. (A) Anti-Zasp antibody (red), (B) anti-talin antibody (green) and (C) merged image of stage 16 embryo. The boxed area in C is shown enlarged in A′–C′. Arrows indicate myotendinous junctions.

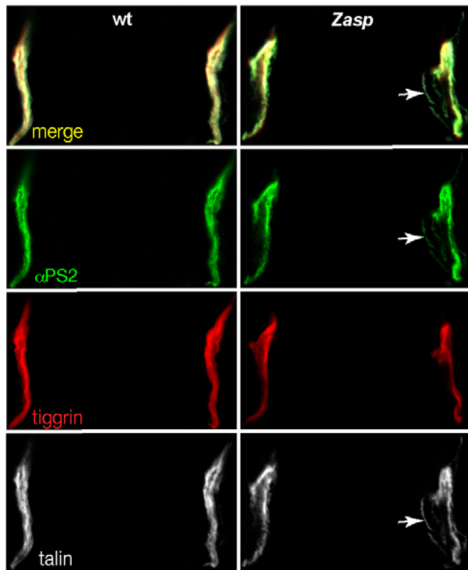

Fig. 3. Integrins detach from the ECM in *Zasp* mutants. Absence of Zasp causes detachment of αPS2 integrin from the *Drosophila* ECM ligand, tiggrin. Comparison of *Drosophila* myotendinous junctions in lateral muscles of wild-type (wt) and *Zasp* mutant embryos. Triple staining with rat anti-αPS2 integrin (green), rabbit anti-talin (white), mouse anti-tiggrin (red), and merged images are shown. In wild-type embryos, tiggrin, αPS2 integrin, and talin colocalize tightly at myotendinous junctions. In contrast, in a *Zasp* mutant embryo, talin and αPS2 integrin (arrows) are partially separated from tiggrin.

that the talin head mutant defective in integrin activation is unable to suppress the *Zasp* mutant phenotype (Fig. 4C), we have provided further genetic support for a role of Zasp in integrin activation.

Zasp cooperates with talin head but not full-length talin to activate integrins

The preceding data establish that Zasp can modulate integrin activation *in vivo* in a talin-dependent manner. Our *in vivo* studies also show that Zasp deficiency does not alter talin localization, suggesting that Zasp may not function by altering talin targeting, as RIAM does (Lee et al., 2009). In cell culture, RIAM cooperates with full-length talin to trigger integrin activation; we therefore asked if Zasp cooperates with full-length talin in CHO cells and compared this activation to the cooperation of talin head and Zasp. Full-length talin is less potent than talin head in activating α5β1 integrins; furthermore, co-expression of Zasp with full-length talin does not potentiate α5β1 integrin activation while Zasp does enhance talin-head-mediated activation (Fig. 5). Hence, unlike RIAM or lamellipodin, Zasp does not recruit nor activate full-length talin in order to trigger integrin activation.

Unlike kindlins, Zasp activates β1 but not β3 integrins

To understand the manner by which Zasp modulates integrin activation, we assessed the specificity of the effects of Zasp on different integrins. While talin plays a central role in the activation of many integrins, previous studies have shown that kindlin acts in an integrin-specific manner, cooperating with talin to activate β3 and β2 but not β1 integrins (Harburger et al., 2009; Ma et al., 2008; Moser et al., 2009a). To compare the effect of Zasp on activation of β1 and β3 integrins, we assessed the effect of Zasp expression on the activation of endogenous α5β1 integrin and stably expressed αIIbβ3 integrin in CHO cells in parallel. αIIbβ3-expressing CHO cells were transfected and α5β1 activation assessed with FN9-11 in the presence of the selective αIIbβ3 antagonist XP-280 (Barrett et al., 1999), or the α5β1-specific antagonist 3F compound (Heckmann et al., 2007). In the same transfected CHO cell population, αIIbβ3 activation was assessed with the activation-specific, ligand-mimetic, anti-αIIbβ3 monoclonal antibody PAC-1 (see Materials and Methods). When co-expressed with talin head, Zasp V1 enhanced talin-head-mediated α5β1 activation (Fig. 6A) but not αIIbβ3 activation (Fig. 6B) compared to talin head alone. Thus, Zasp synergizes with talin head to activate β1 but not β3 integrins. The inability of Zasp to potentiate talin-head-mediated activation of αIIbβ3 integrin was not because the integrins were already maximally activated as, consistent with our previous results (Goult et al., 2009; Harburger et al., 2009), co-expression of kindlin-1 enhanced talin-head-mediated αIIbβ3 activation (Fig. 6B). As we have previously shown, kindlin-1 exerts integrin-specific effects on activation and does not activate α5β1 in CHO cells (Fig. 6A) (Harburger et al., 2009). Thus, Zasp specifically activates β1 integrins and this specificity distinguishes it from the previously characterized kindlin co-activators.

Zasp does not cooperate with kindlin

Our data demonstrate that Zasp is a new integrin co-activator with specificity for β1 integrins. To assess whether Zasp can cooperate with other integrin co-activators, like kindlin, we measured FN9-11 binding to endogenous α5β1 integrin in CHO cells co-expressing kindlin-1 and Zasp. Neither co-expression of kindlin-1 with Zasp V1 nor co-expression of kindlin-1 with DsRed alone led to α5β1 integrin activation (Fig. 6C). Indeed, as previously reported (Harburger et al., 2009), kindlin-1 overexpression inhibits α5β1 activation in CHO cells (Fig. 6C) and Zasp did not alter this effect. Therefore, Zasp does not demonstrate cooperation with kindlin-1 in β1 integrin activation.

Zasp LIM domains bind β1 integrin tails but are dispensable for Zasp-mediated integrin activation

Kindlin co-activation of αIIbβ3 integrin requires binding of the kindlin PTB-like domain to the β3 integrin tail (Harburger et al., 2009; Moser et al., 2008; Ussar et al., 2008). To understand the mechanism by which Zasp co-activates β1 integrin, we tested whether Zasp binds the β1 tail. Using integrin tails immobilized on nickel beads, we pulled down overexpressed Zasp proteins from CHO cell lysates. We demonstrate that full-length Zasp V1 binds the β1 cytoplasmic tail in a specific manner, as control αIIb integrin tails do not pull down Zasp V1 (Fig. 7A), establishing, for the first time, that Zasp is capable of binding integrins. We obtained similar results for *Drosophila* Zasp binding to βPS integrin (supplementary material Fig. S2; data not shown).

To determine which domains of Zasp mediate this interaction, we tested the ability of several Zasp V1 truncations to bind to αIIb and β1 immobilized tails. As shown in Fig. 7A, only truncations containing the LIM domains [Zasp V1 full length, C-terminus (Cter), LIM domains alone] are able to bind specifically to β1. In contrast, truncations lacking the LIM domains [N-terminus (Nter), ΔLIM] are unable to bind β1 integrin. Therefore, our results show that Zasp binds to β1 integrin tails and that the LIM domains of Zasp are required and sufficient for binding.

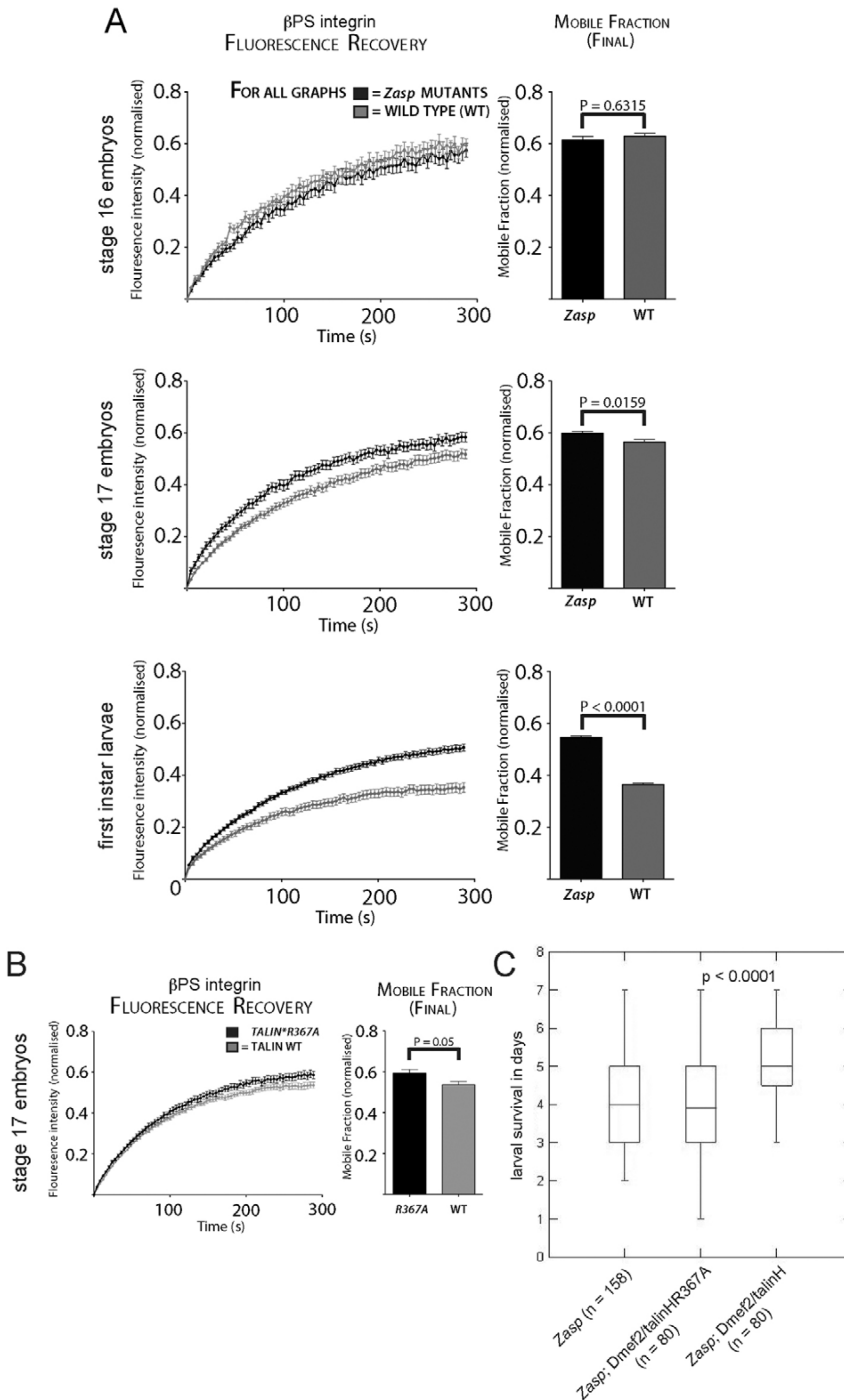

Fig. 4. ***Drosophila*** **Zasp modulates integrin function.** (**A**) FRAP of integrin–YFP at myotendinous junctions from wild-type and *Zasp* mutant embryos at different stages. Integrin mobility decreases only in wild type embryos during maturation of myotendinous junctions. (**B**) FRAP of integrin–YFP at myotendinous junctions from wild-type talin and talinR367A mutant stage 17 embryos. Integrin mobility is higher in talinR367A mutants. (**C**) *T*alin head, but not talin headR367A, can increase larval survival in *Drosophila* when expressed in muscles of *Zasp* mutants.

Talin and kindlin binding to the integrin tail is required for their ability to activate (Bouaouina et al., 2008; Harburger et al., 2009; Tadokoro et al., 2003). To determine whether LIM-mediated binding of Zasp to β1 integrin is required for Zasp co-activation of endogenous α5β1 integrin in CHO cells, we co-expressed the same Zasp truncations with talin head and assessed their ability to co-activate α5β1. Unexpectedly, we found that not all LIM containing Zasp fragments activate α5β1; while Zasp V1 full-length and Cter potentiate talin-head-mediated activation, Nter and the LIM domains alone do not (Fig. 7B). Interestingly, the ΔLIM construct activates despite its inability to bind integrin (Fig. 7A,B). Therefore, LIM-mediated binding of Zasp to β1 integrin is not a condition for Zasp co-activation. We further find that the middle sequence of Zasp V1 (215–546), located between

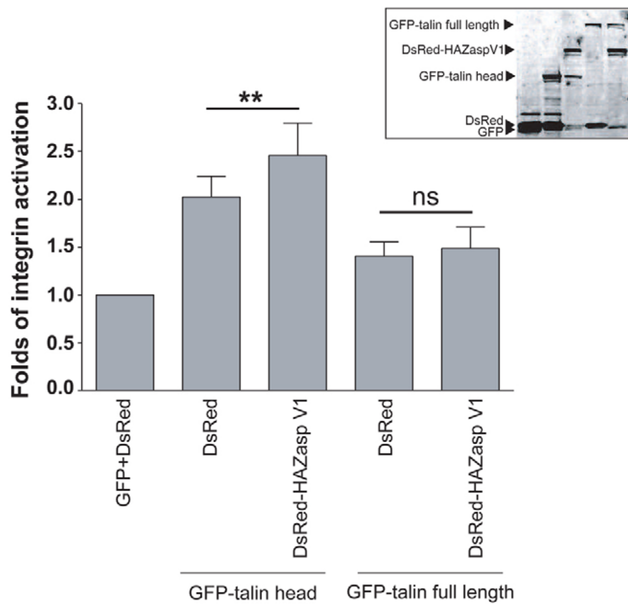

Fig. 5. Human Zasp does not cooperate with full-length talin. CHO cells were co-transfected with GFP, GFP–talin head or full-length GFP–talin and DsRed or DsRed–HAZasp. Full-length talin and Zasp show similar levels of α5β1 integrin activation as full-length talin alone. Activation indices of doubly transfected cells (GFP and DsRed positive) were calculated and normalized for integrin expression. Results represent mean ± s.e.m. ($n \geq 3$; **$P<0.01$). Inset shows expression of transiently transfected proteins.

the ZM and the LIM domains, is required for Zasp-mediated integrin activation. However, co-expression of Zasp V1 (215–546) with talin head fails to activate α5β1 (Fig. 7C), indicating that it is not sufficient. These data confirm that Zasp modulates β1 integrin activation through a novel mechanism distinct from other known integrin co-activators, like kindlin or RIAM, and indicate that LIM-domain-mediated Zasp–integrin interactions are not essential for co-activation by Zasp.

Cardiac- and skeletal-muscle-specific Zasp isoforms modulate integrin activation

Zasp is alternatively spliced in mice and humans and the expression pattern of its resulting isoforms is tissue specific (Arimura et al., 2004; Huang et al., 2003). We have shown that the cardiac-specific isoform Zasp V1 activates β1 integrin. To determine whether another Zasp isoform containing the middle fragment required for Zasp V1 integrin activation is able to activate β1 integrin, we tested the skeletal isoform Zasp V6 (Arimura et al., 2004) in our cell-based activation assay. When co-expressed with talin head, Zasp V6 activates β1 integrin similarly to Zasp V1 (Fig. 7D), indicating that integrin regulation by Zasp can be mediated by other splice variants and suggesting that regulation may occur in various tissues.

Discussion

Integrin activation is a tightly regulated process important for a variety of cellular activities including cell adhesion, migration and assembly of extracellular matrices. The molecular basis for the conformational changes in integrin extracellular domains that underlie activation is increasingly well understood (Luo et al., 2007), and it is recognized that binding of talin head to integrin β subunit cytoplasmic tails plays a key role in inside-out integrin activation (Moser et al., 2009b; Shattil et al., 2010). However, it is now known that additional factors collaborate with talin to enhance integrin activation. Here, combining *in vivo* studies in *Drosophila* and activation assays in mammalian cell culture, we show that the muscle-specific protein Zasp cooperates with talin head to enhance integrin activation. This conclusion is based on the similarity in phenotypes of Zasp-deficient and talinR367A-mutant *Drosophila*, genetic rescue of the Zasp null phenotype by talin head overexpression, suppression of lethality associated with integrin activating mutations in *Zasp* heterozygous flies, enhanced mobility of βPS integrins in Zasp-deficient muscles and integrin activation in CHO cells. Notably, Zasp potentiates talin-head-mediated activation of α5β1 but not αIIbβ3 integrins, making it distinct from other known integrin co-activators.

Zasp is mutated in cardiomyopathies and myofibrillar myopathies and knockout of Zasp in mice, zebrafish or *Drosophila* leads to severe muscle defects (Jani and Schöck, 2007; Sheikh et al., 2007; van der Meer et al., 2006; Zhou et al., 2001). The ability of muscles to transmit intracellular actomyosin-mediated contractility to neighboring cells and tissues requires adhesion to the ECM and assembly of cytoskeletal complexes that link adhesion receptors to the contractile apparatus (Sparrow and Schöck, 2009). Our *in vivo* data in *Drosophila*, in particular the increased integrin mobility in Zasp and talinR367A mutant myotendinous junctions, demonstrate that Zasp regulates integrin function in muscles and is required for myotendinous junction maturation. The partial rescue of *Zasp* mutants by the overexpression of the talin head domain, and the attenuation of lethality in βPS mutants by removing one allele of Zasp or talin, indicate that Zasp regulates integrin activation in *Drosophila*. Thus, in addition to its previously recognized role in the assembly and maintenance of the muscle contractile machinery, we demonstrate that Zasp may also serve to coordinate muscle adhesion through modulation of integrin activation.

Talin is a well-characterized integrin activator, and it is known that the binding of talin head to integrin β tails triggers integrin activation by disrupting inhibitory interactions between the transmembrane and membrane-proximal regions of the integrin α and β subunits (Moser et al., 2009b; Shattil et al., 2010). Genetic analyses and cell-based studies have identified integrin regulators that act in a talin-dependent manner, such as kindlin, RIAM and lamellipodin (Han et al., 2006; Harburger et al., 2009; Lee et al., 2009; Ma et al., 2008; Montanez et al., 2008; Moser et al., 2009a; Moser et al., 2009b; Moser et al., 2008; Shattil et al., 2010). Our study identifies Zasp as an additional talin-dependent regulator of integrin activation by demonstrating in a mammalian cell-based system that Zasp activates β1 integrins when co-expressed with talin head but not when expressed alone. These data corroborate the genetic interaction between Zasp and talin observed in *Drosophila* and establish Zasp as a new integrin co-activator.

A number of known integrin activators act via full-length talin. For instance, RIAM and lamellipodin act by recruiting full-length talin to the cellular membrane, where it subsequently becomes activated and interacts with integrins (Lee et al., 2009). While Zasp potentiates talin-head-mediated α5β1 activation; we found it has no effect on activation induced by full-length talin. The inability of Zasp to enhance full-length talin-mediated α5β1 activation is consistent with our observation that Zasp

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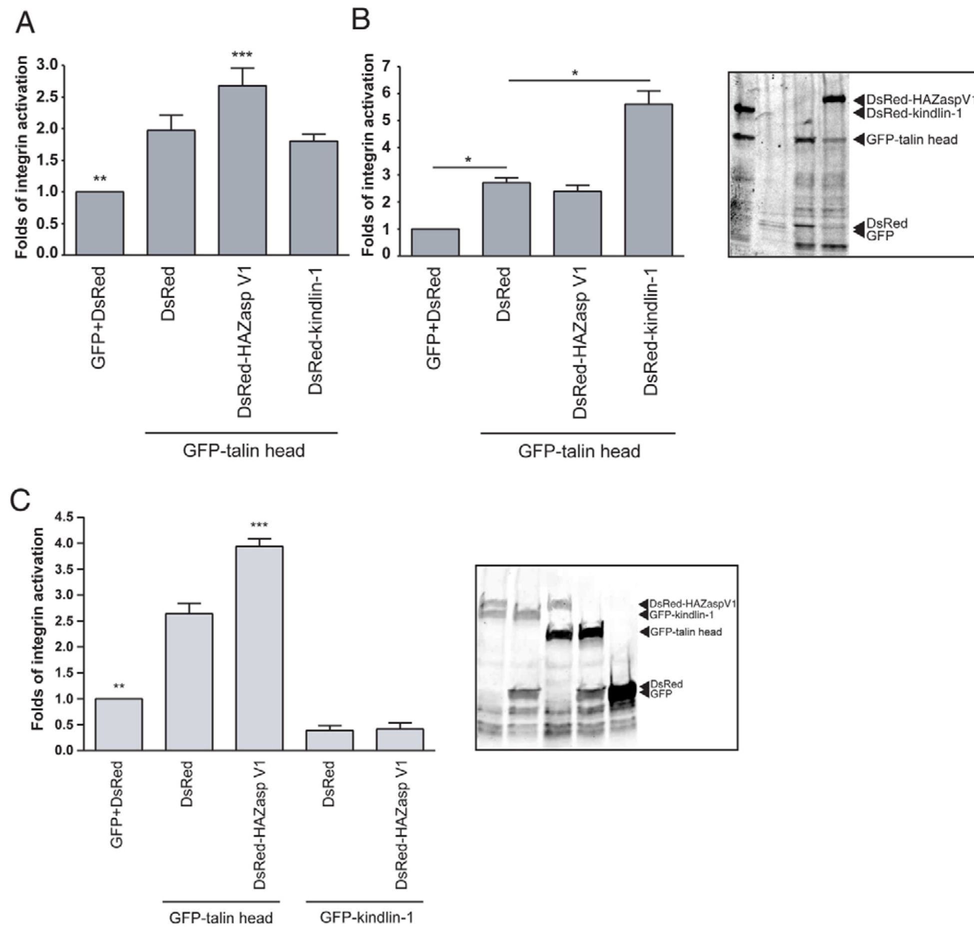

Fig. 6. Human Zasp specifically activates α5β1 but not αIIbβ3 integrins and does not cooperate with kindlin in α5β1 integrin activation. CHO cells stably expressing αIIbβ3 integrin were co-transfected with GFP or GFP–talin head and DsRed, DsRed-HAZasp V1 or DsRed-kindlin-1 constructs. After detachment, cells from each transfection were incubated with either FN9-11 or PB1 antibody (α5β1 integrin activation) or with PAC-1 or D57 antibodies (αIIbβ3 integrin activation) with appropriate antagonists. Cells co-expressing similar amounts of GFP- and DsRed-tagged proteins were analyzed (see Materials and Methods). Activation indices were normalized for integrin expression. Results significantly different from DsRed and GFP–talin-head are indicated. Values are means ± s.e.m. ($n \geq 3$; *$P<0.05$, **$P<0.01$ and ***$P<0.001$). (**A**) Zasp V1, but not kindlin-1, cooperates with talin head to activate α5β1 integrins. FN9-11 binding in the presence or absence of either XP280 (αIIbβ3 antagonist) or 3F compound (α5β1 antagonist) was measured (see Materials and Methods). (**B**) Kindlin-1, but not Zasp, synergizes with talin head to activate αIIbβ3 integrins. Binding of the αIIbβ3-specific, ligand-mimetic, PAC-1 antibody was measured. Inset shows expression of transiently transfected proteins. (**C**) Zasp V1 does not cooperate with kindlin-1 to activate α5β1 integrins. Experimental procedure was conducted as in A. Inset shows expression of transiently transfected proteins.

overexpression has no impact on α5β1 activation in cells expressing endogenous full-length talin. Furthermore, the absence of Zasp in *Drosophila* muscles does not alter talin localization. Together these data show that integrin activation by Zasp occurs in a manner distinct from that of RIAM or lamellipodin, and suggest that Zasp collaborates with the activated form of talin, perhaps by increasing the affinity of talin head for integrin cytoplasmic tails. These data also suggest that Zasp is not sufficient, by itself, to activate endogenous or overexpressed talin.

The kindlins have also emerged as conserved regulators of integrin activation (Bouaouina and Calderwood, 2011; Meves et al., 2009; Ye and Petrich, 2011). However, while kindlins play an important role in muscle attachment in *Drosophila*, it is not known whether they impact βPS integrin activation (Bai et al., 2008), and despite considerable recent interest in kindlin function, the molecular basis for the effect of kindlins on mammalian integrins remains unknown. Kindlin-mediated integrin activation appears to be talin dependent and integrin specific; co-expression of kindlin-1 or -2 with talin head enhances αIIbβ3 activation (Ma et al., 2008; Montanez et al., 2008; Moser et al., 2009a; Moser et al., 2009b; Moser et al., 2008; Shattil et al., 2010), but kindlin-1 and -2 do not activate α5β1 integrins (Harburger et al., 2009). Further, the loss of the hematopoietic kindlin-3 affects β2 and β3 (Manevich-Mendelson et al., 2010; Moser et al., 2009a) but not β1-mediated cell adhesion (Manevich-Mendelson et al., 2010). Here we show that unlike kindlins, which co-activate β3 integrins, Zasp specifically co-activates β1 and not β3 integrins. Our cell-based integrin activation assay monitors the expression of each integrin activator, employs integrin-specific antagonists and uses activation-specific reporters, allowing us to assess Zasp specificity toward both endogenous α5β1 and stably expressed αIIbβ3 integrins in the same transfected CHO cells. We further

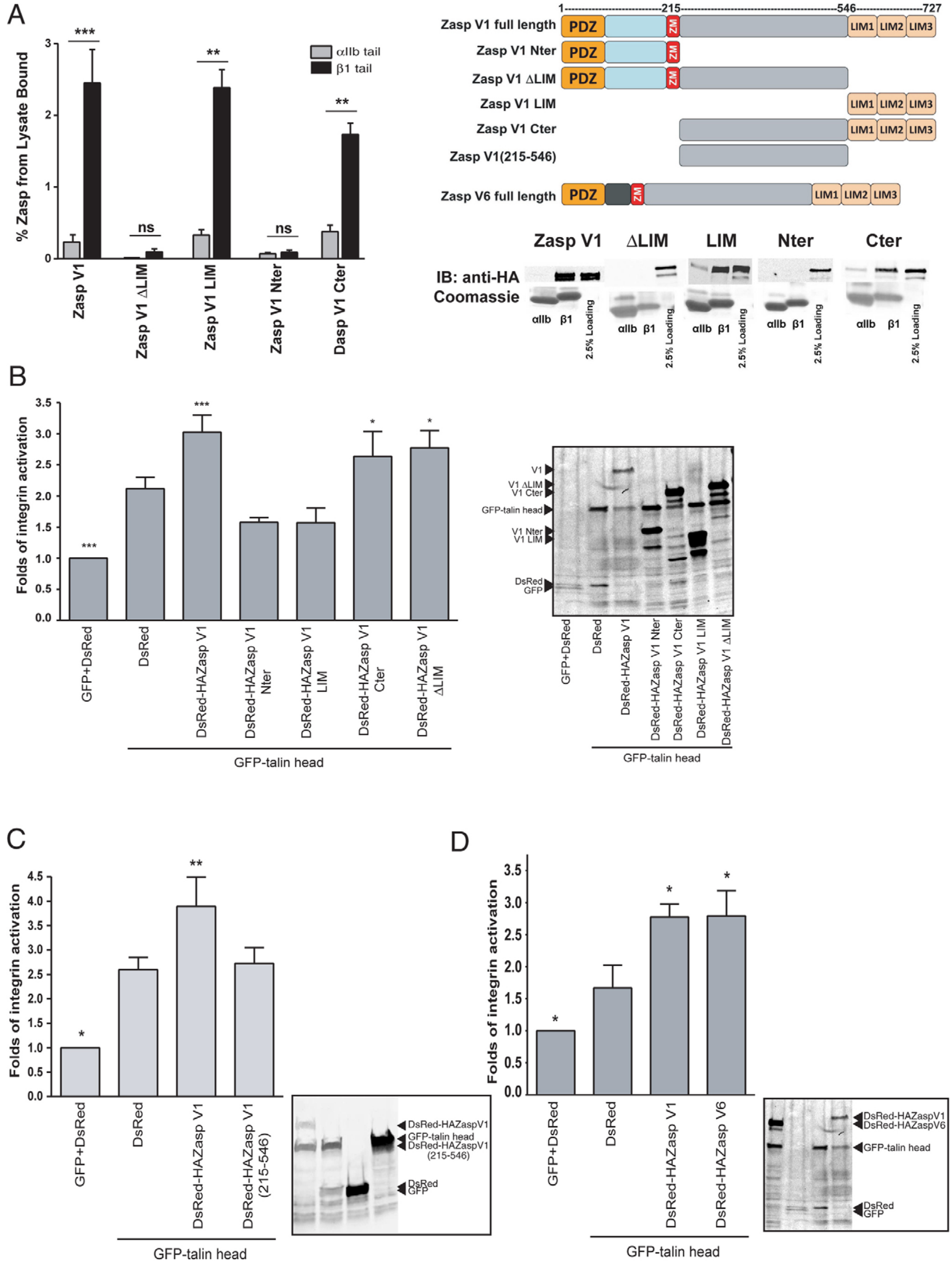

Fig. 7. See next page for legend.

find that Zasp does not cooperate with kindlin in our activation assays. Thus, the activating effects of Zasp have integrin specificity distinct from other currently known activators.

Some integrin activators, such as kindlins, exert their activation functions through binding to the integrin tail. Kindlins bind integrin β tails directly, and mutations that interfere with kindlin–integrin interactions abolish the activating effect of kindlin (Harburger et al., 2009; Ma et al., 2008; Montanez et al., 2008; Moser et al., 2009a; Moser et al., 2009b; Moser et al., 2008). Here, we report that Zasp binds the integrin β1 tail through its C-terminal LIM domains. Unexpectedly, we find that Zasp-mediated integrin activation occurs independently from Zasp binding to the integrin tail, reinforcing our understanding that Zasp is acting in a manner distinct from kindlins. The role of the Zasp–integrin binding remains unclear; however, other muscle LIM-containing proteins like the four and one-half LIM domain proteins (FHL-2 and -3) were shown to bind to the α7β1 integrin in muscles (Samson et al., 2004; Wixler et al., 2000) while their binding is dispensable for cell adhesion (Chu et al., 2000). It is possible that integrin binding of Zasp serves to tether Z-lines to the sarcolemma through the costamere, which is consistent with recent data showing that long isoforms of Zasp/Cypher are present in the non-filament fraction of heart tissue together with β1D integrin, whereas the short isoform lacking LIM domains was present only in the filament fraction (Cheng et al., 2010).

The region of Zasp required for activation (V1 215–546) does not contain any predicted domains, and sequence analysis indicates a highly disordered region common to both activating Zasp isoforms (V1 and V6). Given the inability of this region alone to cooperate with talin head, it is possible that the PDZ-ZM or the LIM domains are required to stabilize it structurally and/or to provide a means of targeting to integrin rich sites, potentially allowing the middle portion of Zasp to act as an adaptor/docking region involved in integrin activation signaling.

In summary, we identify Zasp as a novel regulator of integrin activation, with selectivity for β1 over β3 integrins and requiring its middle region (V1 215–546) in order to co-activate. We further demonstrate for the first time that Zasp is capable of binding β1 tails. While the mechanism of action for Zasp remains unknown, our data reveal that Zasp acts in a manner distinct from known integrin co-activators. Many other PDZ–LIM proteins also have been shown to modulate cell attachment and migration, such as Mystique, which is expressed in the lungs and co-localizes with β1 integrins (Loughran et al., 2005). Given that mammals have 10 genes encoding PDZ–LIM domain proteins (Alp/Enigma family plus LMO7, LIMK1, and LIMK2), with varying tissue specificity, it is possible that a variety of other, currently unrecognized proteins may also contribute to the fine-tuning of integrin activation.

Fig. 7. Human Zasp binding to the β1 tail is not required for integrin activation and the activating function of Zasp is conserved between isoforms. (**A**) Zasp binding to the β1 tail is LIM-domain mediated. Pulldown assays using recombinant αIIb or β1 tail proteins were performed with transfected CHO cell lysates expressing full-length DsRed–HAZasp V1 or DsRed–HAZasp V1 fragments. Binding was quantified by densitometry and normalized to the lysate control ($n \geq 3$; $^{**}P<0.01$, $^{***}P<0.001$). Upper right: schematic of the domain organization of Zasp fragments and isoforms. Lower right: representative pulldowns of overexpressed DsRed-HAZasp V1 constructs. Binding was assessed by western blotting (anti-HA tag). Loading of each tail protein was judged by protein staining. Loading control represents 2.5% of the starting lysate in the binding assay. (**B**,**C**) Zasp-mediated integrin activation requires Zasp V1 middle region (215–546) but does not rely on integrin binding. CHO cells were co-transfected with GFP or GFP–talin head and DsRed, DsRed–HAZasp V1 constructs. Activation indices of α5β1 integrin from cells co-expressing similar amounts of GFP- and DsRed-tagged proteins were calculated and normalized for integrin expression (see Materials and Methods). Values are means ± s.e.m. ($n \geq 3$). Results that are significantly different from DsRed and GFP–talin head ($^{*}P<0.05$, $^{***}P<0.001$) are indicated. Insets show expression of transiently transfected proteins. (**D**) The skeletal-specific isoform Zasp V6 activates integrin. CHO cells were co-transfected with GFP or GFP–talin head and DsRed, DsRed–HAZasp V1 or V6 constructs. Experimental procedure was as in C. Inset shows expression of transiently transfected proteins.

Materials and Methods

Drosophila genetics and antibody stainings

Fly strains used were *Zasp* (Jani and Schöck, 2007), *rhea*^{79}, *rhea*^{2B} (provided by N. H. Brown, University of Cambridge, Cambridge, UK) (Brown et al., 2002), UAS-talinHead, UAS-talinHeadR367A, pUbi-talinWT, pUbi-TalinR367A (Ellis et al., 2011; Tanentzapf and Brown, 2006; Tanentzapf et al., 2006; Yuan et al., 2010), *mys*^{b30}, *mys*^{b44} (provided by T. A. Bunch, Arizona Cancer Center, Tucson, AZ, USA) (Kendall et al., 2011) and *Dmef2-Gal4* from the Bloomington *Drosophila* Stock Center. For Fig. 4B, the pUbi-βPSYFP *rhea*^{79} recombinant was generated by standard genetic crosses. The genotypes were: pUbi-talinR367A/+; pUbi-βPSYFP *rhea*^{79}/*rhea*^{79} and pUbi-talinWT/+; pUbi-βPSYFP *rhea*^{79}/*rhea*^{2B}. For Fig. 4C, eggs were collected on apple juice plates for 4 hours at 25℃ and aged for 18 hours. Embryos were dechorionated in 50% bleach for 90 seconds and transferred to new apple juice plates. Viability was assessed by daily counts of surviving larvae. For the genetic interaction with integrins, we crossed *mys*^{b44}/FM7c and *mys*^{b30}/FM7c to *Zasp*/CyO at the appropriate temperature. Mutant viability on its own was determined by calculating the ratio of *mys*^{b44}/Y; +/CyO and *mys*^{b44}/+; +/CyO. Mutant viability with removal of one copy of *Zasp* was determined by the ratio of *mys*^{b44}/Y; *Zasp*/+ and *mys*^{b44}/+; *Zasp*/+. For antibody stainings, embryos were heat-fixed by immersion in boiling embryonic wash buffer (70 mM NaCl and 0.05% Triton X-100) for 10 seconds, immediately cooled by adding three volumes of ice-cold embryonic wash buffer, and placed on ice for 30 minutes. Embryos were then devitellinized in methanol/heptane. Antibody staining was performed and images obtained as described previously (Jani and Schöck, 2007). Primary antibodies used were: rabbit anti-Zasp antibody (1:200) (Jani and Schöck, 2007), rat anti-αPS2 integrin (1:10; 7A10; provided by N. H. Brown) (Brower et al., 1984), rabbit anti-talin (1:100; provided by N. H. Brown) (Brown et al., 2002), and mouse anti-tiggrin (1:300; provided by J. H. Fessler, University of California Los Angeles, USA) (Fogerty et al., 1994). Secondary antibodies were of the Alexa Fluor series (1:400; Invitrogen).

Drosophila βPS integrin binding assays

βPS integrin cytoplasmic tail peptides were diluted to 20 µg in 200 µl PBS and used to coat a 96-well plate (Corning) overnight at 4℃. Wells were blocked with 1% BSA and incubated with embryo extracts or purified GST fusion proteins for 1 h at 37℃. Wells were incubated again with 1% BSA for 10 minutes, and washed three times for 15 minutes in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40 and EDTA-free complete protease inhibitor cocktail from Roche Diagnostics). Bound protein was then immunoblotted by standard procedures with rabbit anti-Zasp antibody (1:5000) or rabbit anti-GST (1:100, Santa Cruz). GST-Zasp^{isoform10FL} (1–780) was cloned from EST RH03424 into pGEX-5X-1 (GE Healthcare), then overexpressed and purified by standard procedures.

Fluorescence recovery after photobleaching and statistical analysis

FRAP experiments were carried out as described (Yuan et al., 2010). Briefly, experiments were performed on embryos and larvae collected from apple juice plates. Embryos were dechorionated using a 50% bleach solution for 4 minutes and rinsed in dH_2O. Larvae were rinsed in PBS. The samples were then mounted live on glass slides in PBS. FRAP experiments were carried out 2 hours after mounting at room temperature using an Olympus FV1000 laser scanning confocal microscope. Fluorescence was bleached using a 405 nm laser for 2 seconds at 30% power at 100 µs/pixel. The recovery of fluorescence was imaged through a UplanSApo 60×/1.35 NA oil objective every 4 seconds for a total of 75 frames. Statistical analysis was performed with GraphPad Prism (GraphPad Software).

Mammalian antibodies and expression constructs

Mouse anti-HA (Covance), goat anti-DsRed (Santa Cruz) and goat anti-GFP (Rockland) antibodies were purchased. Anti-hamster α5β1 PB1 developed by

Brown and Juliano (Brown and Juliano, 1985) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa. GFP-tagged mouse talin head (amino acids 1–433) was previously described (Bouaouina et al., 2008). DsRed-HAZasp V1 was generated by PCR from a human Zasp V1 clone obtained from ATCC (Clone ID 40080656) and subcloned in frame with an N-terminal DsRedMonomer-HA tag. DsRed–HAZasp V6 was generated by subcloning the skeletal-specific region from a cDNA clone from ATCC (Clone ID 4291498) into DsRed-HAZasp V1. DsRed-HAZasp V1 ΔLIM (amino acids 1–546 lacking three C-terminal LIM domains), DsRed-HAZasp V1 LIM (540–727) (all three LIM domains), DsRed-HAZasp V1 Cter (215–727; missing all the three LIM domains), DsRed-HAZasp V1 Nter (including the PDZ and ZM) and DsRed-HAZasp V1 (215–546) fragment were generated by PCR from DsRed-HAZasp V1 full length then all were confirmed by DNA sequencing.

Analysis of integrin activation

The activation states of α5β1 and αIIbβ3 integrins in CHO cells were assessed by three-color flow cytometric assays as described previously (Bouaouina et al., 2012). α5β1 activation was assessed by measuring the binding of a recombinant soluble integrin-binding fragment of fibronectin (FN9–11). The activation state of stably expressed αIIbβ3 integrins was assessed by measuring the binding of the activation-specific, ligand-mimetic monoclonal antibody PAC-1. Briefly, CHO cells were transfected with the indicated cDNAs using either Lipofectamine (Invitrogen) or PEI (linear polyethyleimine 25 kDa, Polysciences Inc.). After 24 hours, cells were resuspended and activation of α5β1 and αIIbβ3 integrins was assessed in parallel in doubly transfected (GFP-positive and DsRed-positive) cells using an LSRII Flow Cytometer (BD Biosciences).

Assessment of αIIbβ3 integrin activation

PAC-1-specific binding to αIIbβ3 was assessed in the absence or presence of the αIIbβ3-specific antagonist XP280; a gift from Dr Shaker A. Mousa (Barrett et al., 1999). Total αIIbβ3 integrin expression levels were measured in parallel by staining with D57 (O'Toole et al., 1994). After washing cells, bound PAC-1 and D57 were detected with Alexa-Fluor-647-conjugated goat anti-mouse IgM or goat anti-mouse IgG (Invitrogen), respectively.

Assessment of α5β1 integrin activation

To ensure specific FN9-11-binding to α5β1 in the presence of overexpressed αIIbβ3 integrin, the assay was conducted in the presence or absence of two specific integrin antagonists. To block background FN9-11 binding to αIIbβ3, XP280 was added to cells incubated with FN9-11 in the presence or absence of EDTA. Alternatively, an α5β1-specific inhibitor, 3F, kindly provided by Dr Horst Kessler (Heckmann et al., 2007), was used to specifically block FN9-11-binding to α5β1. Total α5β1 integrin expression levels were measured in parallel by staining with PB1 (Brown and Juliano, 1985). After washing cells, bound FN9-11 and PB1 were detected with Allophycocyanin (APC)-conjugated streptavidin (Thermoscientific) or Alexa-Fluor-647-conjugated goat anti-mouse IgG, respectively. The effective concentration of all reporters and antagonists was determined by titration.

Calculation of integrin activation indices

Activation index was defined as $AI=(F-F_o)/(F_{integrin})$. For αIIbβ3, F is the geometric mean fluorescence intensity (GMFI) of PAC-1 binding; F_o is the GMFI of PAC-1 binding in the presence of XP280. For α5β1, F is the GMFI of FN9-11 binding and F_o is the GMFI of FN9-11 binding in the presence of 3F compound. Alternatively, F is the GMFI of FN9-11 in presence of XP280 binding and F_o is the GMFI of FN9-11 binding in the presence of XP280 and EDTA. $F_{integrin}$ is a standardized expression ratio of PB1 or D57 binding to transfected cells, defined as: $F_{integrin}=(F_{trans})/(F_{untrans})$, where F_{trans} is the GMFI of PB1 or D57 binding to doubly expressing cells, and $F_{untrans}$ is the GMFI of PB1 or D57 binding to untransfected cells. FACS data analysis was carried out using FlowJo analysis software and statistical analysis using Student's *t*-test was performed by GraphPad Prism software.

Integrin pull-down assays

Integrin pull-down assays were performed as previously described (Harburger et al., 2009; Lad et al., 2007). Briefly, Chinese hamster ovary (CHO) cells were seeded on 10 cm tissue culture dishes and transiently transfected using Lipofectamine (Invitrogen) or polyethylenimine (Polysciences). After 24 hours, cells were harvested and lysed. Cell lysates were cleared by centrifugation then incubated with integrin tails bound to beads overnight at 4℃. The beads were washed, resuspended in SDS sample buffer, heated for 5 minutes at 95℃ and run on 4–20% Tris–glycine SDS-polyacrylamide gradient gels (Bio-Rad). Loading of integrin tails was assessed by Coomassie Blue staining. Pulldown was assessed by western blotting (mouse anti-HA antibody). Bands were quantified using ImageJ and normalized to 2.5% loading control. Statistical analysis using Student's *t*-test was performed using GraphPad Prism.

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Journal of Cell Science

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